PATENT SPECIFICATION (11) 1 343 113

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(21) Application No. 22342/70 (22) Filed 8 May 1970
(23) Complete Specification filed 11 Nov. 1970
(44) Complete Specification published 9 Jan. 1974
(51) International Classification C07C 103/52
A61K 27/00
(52) Index at acceptance
C2C 171—191—280 172—192—282 172—193—283 174—177—181 17X—191—278 183—193—277 214 215 220 225 226 22Y 246 247 248 250 251 252 256 258 25Y 28X 30Y 311 31Y 320 321 32Y 341 342 34Y 350 351 352 355 360 361 362 365 366 367 368 36Y 380 387 491 576 620 626 628 630 635 645 64X 658 678 67X 694 699 72X 738 750 753 754 75X 761 763 76X 78Y 790 79Y KD KZ SM TA TP

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(54) IMPROVEMENTS RELATING TO INSULIN DERIVATIVES

(71) We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British Corporation established by Statute, of Kingsgate House, 66 - 74 Victoria Street, London, S.W.1., do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to insulin derivatives.

Porcine and bovine insulin have been used clinically for many years in the treatment of diabetes and other disorders. One of the disadvantages of these materials however is that for certain patients it is necessary after treatment has been continued for some time to increase the dose in order to produce the required effect. This has been explained by ascribing antigenic properties to these non-human insulins which give rise to antibodies in sufficient amount to counteract a proportion of the dose of insulin applied.

It has now been found possible to reduce considerably and perhaps even eliminate the ability of porcine and bovine insulins to react with specific insulin antibody without unduly affecting the desirable properties of these hormones.

In accordance with this invention physiologically acceptable substantially pure mono-, di- or tri-substituted insulin derivatives having reduced reactivity to insulin antibodies are those in which the terminal amino group of the B chain (B_1 phenylalanine) is protected by an acyl group or other blocking group containing up to 7 carbon atoms and the amino group of the A chain (A_1, glycine) is either free or protected by means of an acyl or other blocking group containing no more than four carbon atoms and no free primary amino group. The blocking groups in each case may be straight or branched chain or cyclic radicals containing carbon and if desired other elements including, for example, oxygen, nitrogen and sulphur. The term "pure" is used herein to mean free from by products of manufacture.

Although insulin has been subjected to many substitution reactions in the past the results recorded in the literature are complex and difficult to interpret due chiefly to the fact that such reactions give rise to a complex mixture of products and unless proper care is taken in the separation and purification of various products, it is impossible to reach firm conclusions as to the biological properties of the derivatives so formed. Most workers have reported a substantial loss in activity and it has not been recognised, prior to the present invention, that by careful selection of the substituent protective groups derivatives may be produced which have blood sugar lowering properties very similar to that of the parent insulin, at least as regards the initial effect upon intravenous injection into experimental animals, but have sugnificantly reduced reactivity to wards specific insulin antibodies. To achieve these effects the nature and size of the protective group on the B chain in the compounds of the present invention is not as critical as that on the A chain and it will be appreciated that different blocking groups may be used for the respective amino groups. However in practice it is considerably more convenient to use the same blocking group for both amino groups, and also for the third amino group of the B.29 amino acid (lysine) where tri-substituted derivatives are formed.

Protection of the desired amino groups is readily achieved by acylation. Subject to the qualifications expressed above with regard to the protective groups, a wide variety of acylating agents may be used to introduce groups such as formyl, acetyl, trifluoroacetyl, hydroxypropionyl, cyclopropane-

[Price 25p]

carbonyl, aceto-acetyl and other aliphatic acyl groups, benzoyl and other aroyl groups as well as those derived from certain heterocyclic compounds e.g. 2,2-dimethyl-3-formyl-L-thiazolidine-4-carboxylic esters. Acylation may be conducted by any of the standard methods employed in peptide chemistry including especially the use of activated esters or anhydrides and typical carbodiimide coupling reagents. Acylating agents which are esters of N-hydroxysuccinimide are particularly advantageous. A variety of alternative blocking groups include those which introduce the carbamyl, thiocarbamyl, alkyl carbamyl and alkyl thiocarbamyl groups, amidino groups, alkyl.C(=NH).NH, and the guanidino group, $NH_2.C(=NH).NH$. The amidino group $CH_3.C(=NH).NH$ for example, is introduced by means of ethyl acetamidate hydrochloride and the guanidino group by means of O-methyl isourea. Other blocking froups are, for example, $HOCH_2CH_2CO$- (introduced by butyrolactone) $HOOC.CH_2CO$- and the corresponding group $H_2NOC.CH_2CO$- (introduced by the corresponding activated ester of malonic acid or malonamide). The use of more complex acylating and other groups may introduce more difficult separation problems and it is believed that simple acetylation will in practice be most attractive and conducting the reaction to produce the greatest yield of the triacetyl (A_1, B_1, B_{29} substituted), derivative is a particularly recommended procedure in accordance with this invention.

In order to produce the maximum yield of insulin acylated at the B_1 amino group the proportion of acylating agent or other blocking reagent used is preferably relatively low. For example, reacting one mole of the insulin with from about one to not more than about two moles of acylating agent produces the B_1 mono-substituted derivative in the largest amounts. Mono-substitution at other amino groups can however also occur leading to byproducts which are less useful. Furthermore, after substitution at B_1 a certain degree of di-substitution and even tri-substitution can take place especially when three to four moles of acylating or other reagent are used. It has also been found that the acylation reaction depends on the pH of the reaction medium. To produce the best yield of mono-substitution product at the B_1 amino group, the pH is preferably at or near about 7.0 and preferably no greater than about 8. At pH 8.5 to 9 for example the yield of this desired product falls off considerably in favour of additional substitution at A_1 and B_{29}. Usually it will be desirable to isolate the required derivative by means of chromatography. electrophoresis, or any other conventional method of purifying peptides amenable to use on a large scale.

Insulin derivatives in accordance with this invention may be formulated as pharmaceutical preparations in the same way as the parent insulins and may be used clinically at comparable and even lower dosage levels. Thus the desired derivative, after fractionation of the reaction mixture and removal of excess salts e.g. by dialysis, may be recovered in solution form or solid form e.g. by freeze-drying following which it may be made up to the required concentration in an injectable physiologically acceptable diluent such as sterile pyrogen-free water or saline containing suitable buffers, and dispensed in ampoules.

Examples of typical derivatives produced in accordance with the invention will now be described.

EXAMPLE 1

(a) *N-Hydroxysuccinimide ester of 2,2-dimethyl - 3 - formyl - L - thiazolidine - 4-carboxylic acid*

3 - Formyl - 2,2 - dimethyl - L-thiazolidine-4-carboxylic acid (9.46 g., 0.05 moles) was dissolved in redistilled dimethyl-formamide (50 ml.) and the solution cooled to 4°. Recrystallised N-hydroxy-succinimide (5.76 g., 0.05 moles) was added followed by N,N'-dicyclohexylcarbodiimide (10.32 g., 0.05 moles) and the solution stirred overnight at 4°, and for a further two hours at 25°. The dicyclohexylurea formed was filtered off and the dimethylformamide removed on a rotary evaporator under reduced pressure. The additional precipitate of dicyclohexylurea produced was filtered off and the urea was washed with dichloromethane. Petroleum ether (60° - 80°) was added to the supernatant until the solution went turbid. Filtration of the crystalline material produced on standing after drying in a dessicator containing sodium hydroxide and paraffin wax, gave colourless needles of the N-hydroxysuccinimide derivative (13.7 g., 95.7%) m.p. 98 - 100°.

(b) *Reaction of insuline with N-hydroxysuccinimide ester of 2,2-dimethyl-3-formyl-L-thiazolidine-4-carboxylic acid*

Zinc insulin (500 mg., .085 mM) (porcine) ten times recrystallised (Novo Industri A/S, Copenhagen) was dissolved in hydrochloric acid (45 ml., 0.1N) and the pH adjusted with N sodium hydroxide to 6.9 in the titration vessel of a Radiometer (Type TTT1) pH- stat. The hydroxysuccinimide ester (22 mg., 0.085 mM) was dissolved in dioxane (100 μl) and added to the above solution in 10 μl aliquots, maintaining the pH at 6.9 by the addition of alkali, every thirty minutes.

After the final addition of the hydroxy-

succinic ester the reaction mixture was made 0.2 M in sodium carbonate-sodium bicarbonate buffer pH 9.5 and left overnight. The material was lyophilised, after extensive dialysis against distilled water, to yield 490 mg. of the modified protein.

In a second experiment, zinc insulin (500 mg. .085 mM) was treated with the hydroxysuccinic ester (32 mg., 0.127 mM) at pH 8.5 exactly as described above. The reaction rate as judged by the uptake of alkali was much faster than at pH 6.9 and aliquots were added every five minutes.

(c) *Separation of thiazolidine insulins*

(i) *Chromatography*

The thiazolidine insulins were separated on a column of DEAE-Sephadex A-25 (2.5 × 40 cm.). "Sephadex" is a registered Trade Mark.

The column was equlibriated with buffer containing 0.01 M tris and 0.05 M sodium chloride in 7 M de-ionised urea at pH 7.29. Thiazolidine modified insulin was dissolved in this buffer (500 mg., 50 mg./ml.) and the column developed at a flow rate of 54.0 ml./hour collecting 12 ml. fractions. After 120 ml. of eluant was collected, a linear gradient, obtained by running 0.01 M tris, 0.01 M sodium chloride in 7 M de-ionised urea (11), at pH 7.29 into the stirred reservoir of the starting buffer, (11) was applied.

The protein concentration was determined from the absorbance of the solution at 277 mμ.

The appropriate fractions were pooled and extensively dialysed against distilled water. The material was lyophilised and the residue de-ionised on a column (1.5 × 60 cm.) of G.10 Sephadex (Trade Mark) equilibrated with 5% acetic acid. The protein fractions were collected and lyophilised.

The chromatographic separation of the insulin derivatives formed from the above reaction can be seen in Figure 1. Using a gentle salt gradient of 0.05 to 0.1 molar sodium chloride, incomplete separation of the peaks A, B and C occurred. Material under peaks A, B and C was combined and rechromatographed under exactly the same conditions to give the elution pattern shown in Figure 2. Material about the centre of each peak was pooled and each individual peak subjected to rechromatography to give three chromatographically homogeneous fractions. The derivatives corresponding to peaks A, B and C were shown to be mono-substituted on phenylalanine, glycine and lysine respectively.

The elution pattern of the derivatives obtained when insulin was treated with a 1.5 molar excess of the acylating agent at pH 8.5 can be seen in Figue 3. A much simpler elution pattern was obtained and material corresponding to derivative A in the previous chromatography was present in low amount.

(ii) *Cellulose acetate electrophoresis*

For the separation of the thiazolidine insulins, the conditions were those described by Carpenter and Hayes (Biochemistry, *2* (1963) 1272) using a Shandon electrophoresis tank fitted with a water-cooled plate (20 × 20 cm.) and a power supply of 1 Kv. The buffer used was 0.065 M phosphate, 7 M urea, pH 6.5 and the cellulose acetate strips (20 × 5 cm.) were stained in a 0.2% solution of Ponceau S in 3% acetic acid.

Cellulose acetate electrophoresis showed derivatives A, B and C to be electrophoretically pure components which were mono-substituted by comparison with unsubstituted insulin and diacetoacetyl insulin prepared previously.

EXAMPLE 2

Reaction of insulin with diketen

Zinc insulin (1.0 g.) as in Example 1, was dissolved to a final concentration of 0.167 mM in 0.1 M-HCl (45 ml.) and the pH adjusted with M-NaOH to 6.9 in the titration vessel of a Radiometer (type TTT1) pH-stat. Freshly distilled diketen was added in 5μ portions, the pH being maintained at 6.9 by the addition of alkali. When 1 equiv. of alkali was consumed, after approximately 5 minutese, a sample was removed for ninhydrin analysis (Moore & Stein, 1954). Further portions of diketen were added until the ninhydrin colour yield had decreased by 30%. The amount of diketen added was 40 μl (final concentration 0.49 mM).

The reaction mixture was made 0.2 M in Na_2CO_3-$NaHCO_3$ buffer, pH 9.5 and left overnight. After extensive dialysis against distilled water and freeze-drying, the yield of modified protein was 950 mg.

Chromatographic separation of acetoacetyl insulins

The acetoacetyl insulins were separated on a column of DEAE-Sephadex A.25 (2.5 cm. × 40 cm.). "Sephadex" is a registered Trade Mark.

The column was equilibrated with buffer containing 0.01 M-tris and 0.05 M-NaCl in 7 de-ionised urea adjusted to pH 7.20 with M-HCl. Acetoacetyl insulin (500 mg.) was dissolved in this buffer (50 mg./ml.) and the column was developed at a flow rate of 54.0 ml./hr., 12 ml. fractions being collected. After 120 ml. of eluant was collected a linear gradient, obtained by running 0.01 M-tris-0.15 M-NaCl in 7 M de-ionised urea at pH 7.20 into the stirred reservoir of the starting buffer was applied.

The mono-acetoacetylated insulins were rechromatographed on a column of DEAE-Sephadex A-25 (2.5 cm. × 40 cm.) as described above with the exception that the starting buffer was adjusted to pH 7.30

with M-HCl. A linear gradient was applied by running 0.01 M-tris -0.10 M-NaCl in 7M de-ionised urea at pH 7.30 into the stirred reservoir of the starting buffer.

The protein concentration was determined from the extinction of the solution at 277 nm.

Chromatography results for insulin (500 mg.) treated with a threefold molar excess of diketen can be seen in Figure 4. Four main peaks are discernible. The position of elution of unmodified insulin is shown on the Figure. Component I was mainly unmodified insulin; component IV (80 mg.) was chromatographically homogeneous, requiring a higher salt concentration for elution, and would be expected to be more highly substituted. No further protein was eluted.

EXAMPLE 3

N-Hydroxysuccinimide acetate

Acetic acid (3.0 g. 0.05 mole) was dissolved in dichloromethane (30 ml.) and a solution of N-hydroxysuccinimide (5.76 g., 0.05 mole) in anhydrous dioxan (10 ml.) added. N,N'-dicyclohexylcarbodiimide (10.32 g., 0.05 mole) was added and the reaction mixture was stirred overnight at 4° and for a further two hours at room temperature. The dicyclohexylurea formed was filtered off and the solvent removed on a rotary evaporator under reduced pressure. Petroleum ether (60° - 80°) was added to the supernatant until the solution went turbid. Filtration of the crystalline material and recrystallisation from ethyl acetate gave colourless needles of N-hydroxysuccinimide acetate (7.5 g., 95.5%) m.p. 131 - 4°.

Reaction of insulin with N-hydroxysuccinimide acetate

Zinc insulin (200 mg., 0.038 mmol.) was dissolved in hydrochloric acid (40 ml., 0.1 M) and the pH adjusted with sodium hydroxide to 6.9 in the titration vessel of a Radiometer (Type TTT1) pH-stat. N-Hydroxysuccinimide acetate (6 mg., 0.038 mmol.) was dissolved in dioxane (100 μl) and added to the above solution in 10 μl aliquots maintaining the pH at 6.9 by the addition of alkali every thirty minutes.

The reaction mixture was left overnight and was then dialysed against distilled water and lyophilised to yield 185 mg. of the modified protein.

In a second experiment, zinc insulin (500 mg. 0.085 mmol.) was treated with N-hydroxysuccinimide acetate (15.2 mg. 0.085 mmol.) at pH 8.5 exactly as described above, adding aliquots of the activated ester every five minutes.

In a further experiment, zinc insulin (500 mg., 0.085 mmol.) was treated with the activated ester (40 mg. 0.225 mmol.) at pH 8.5 as above.

Chromatographic separation of the acetyl insulins

The acetyl insulins were separated on a column of DEAE-Sephadex A-25 (2.5 × 40 cm.) by a modification of the method described by Bromer & Chance in Biochim. biophys. Acta *133* 219 (1967).

This column was equilibrated with buffer containing 0.01M tris and 0.05 M NaCl in 7 M de-ionised urea adjusted to pH 7.30 with M HCl. The acetyl insulins were dissolved in this buffer (50 mg./ml.) and the column was developed at a flow rate of 54.0 ml./hr., 9.7 ml. fractions being collected. 97 ml. of eluant was collected before a linear gradient was applied. For insulin treated with a molar equivalent of N-hydroxysuccinimide acetate, a linear gradient obtained by running 0.01 M tris and 0.10 NaCl in 7 M de-ionised urea (1 l.) at pH 7.30 into the stirred reservoir of the starting buffer (1 l.) was applied.

For insulins treated with a three-fold molar excess of N-hydroxysuccinimide acetate, the concentration of NaCl in the final buffer was increased to 0.15 M.

The protein concentration was determined from the extinction of the solution at 277 nm.

Tubes around the centre of each peak from the chromatography were pooled and separately rechromatographed under the same conditions. This process was repeated until each peak appeared homogenous by iso-electric focussing. The material was lyophilised and the residue de-ionised on a column (1.5 × 60 cm.) of Sephadex G.10 equilibrated with 5% acetic acid. The fractions were pooled and the protein lyphолised.

At low reagent concentrations and near neutral pH, the predominant products of the reaction are the two mono-substituted acetyl insulins in which the two terminal α-amino groups are modified (Figure 5). However, if the pH is raised the amount of the Phe^{B1}acetyl insulin isolated is reduced and Lys^{B29} acetyl insulin is also produced (Figure 6).

The chromatographic separation of the products of the reaction of insulin with a threefold excess of the activated ester at pH 8.5 can be seen in Figure 7. Characterisation of the two di-substituted acetyl insulins formed shows that they are Phe^{B1} Gly^{A1}-diacetyl insulin and Gly^{A1} Lys^{B29}-diacetyl insulin respectively.

Biological assay

The insulin derivatives were separated from trace amounts of urea and salt by elution from a Sephadex G.10 column (2.5 × 60 cm.) equilibrated with 5% aqueous acetic acid and the protein, after lyophilisation, was dried over phosphorous pentoxide at room temperature for two days. The

derivatives were assayed against a neutral insulin solution (Nuso insulin batch A 354) as standard by the Mouse Convulsion method (British Pharmacopoeia, 1968). All injections were of solutions in acetate buffer, pH 8.0.

EXAMPLE 4

The procedures described in Examples 1, 2 and 3 were repeated using bovine insulin in place of porcine insulin. Virtually identical results were obtained in all cases.

EXAMPLE 5

Following the procedures described in Example 1(b) and 1(c), the monocarbamyl, mono-methylcarbamyl and monomethylthiocarbamyl derivatives of bovine insulin were prepared by the reaction with potassium cyanate and methyl isocyanate and methyl isothiocyanate respectively and subsequently separated by chromatography. The desired substituted derivatives of the B_1 amino acid and other residues were isolated and dialysed to remove excess salts and freeze dried.

EXAMPLE 6

Following the procedure of Examples 1(b) and 1(c), insulin was reacted with methyl acetimidate hydrochloride and the resulting products were worked up as described previously.

WHAT WE CLAIM IS:

1. A physiologically acceptable substantially pure mono-, di- or tri-substituted insulin in which the terminal amino group of the B chain (B_1 phenylalanine) is protected by an acyl or other blocking substituent containing up to 7 carbon atoms and the terminal amino group of the A chain (A_1 glycine) is either free or protected by an acyl or other blocking substituent containing no more than 4 carbon atoms and no free primary amino group.

2. An insulin derivative according to Claim 1, in which the B_1 amino group is protected by an aliphatic acyl group.

3. An insulin derivative according to Claim 2, in which the acyl group is acetyl or acetoacetyl.

4. An insulin derivative according to Claim 1, in which the B_1 amino acid is protected by the 2,2-dimethyl-3-formyl-L-thiazolidine-4-carboxylic acyl group.

5. An insulin derivative according to Claim 1, in which at least one of the substituents is a carbamyl group.

6. An insulin derivative according to Claim 1 being a mono-substituted derivative.

7. An insulin derivative according to Claim 1, being a di-substituted derivative.

8. An insulin derivative according to Claim 1, being a tri-substituted derivative.

9. An insulin derivative according to Claim 7 or 8, in which the two or three protective groups, respectively, are the same.

10. A pharmaceutical preparation containing an effective amount of insulin derivative according to Claim 1 and a physiologically acceptable diluent.

11. A pharmaceutical preparation according to Claim 10, being an injectable aqueous solution of the insulin derivative.

12. A process for producing a compound according to Claim 1 which comprises reacting an insulin with an acylating agent or other blocking reagent to acylate or otherwise block the amino group of the B_1 amino acid and optionally block the A_1 and/or B_{29} amino acid with substituent groups as defined in Claim 1, and in which a mono-substituted, di-substituted or tri-substituted derivative is separated from the reaction mixture.

13. A process according to Claim 12, in which acylation is conducted with from 1 to 2 moles or 3 to 4 moles of acylating agent per mole of insulin.

14. A process according to Claim 13, in which the pH of the reaction medium is maintained at from 7 to 8.5 or 9.

15. A process according to Claim 12, in which the derivative is separated from the reaction medium by means of chromatography, electrophoresis, or other known method of fractionation and the resulting fraction is treated, if desired, to remove salts e.g. by dialysis and the resulting solution of the desired derivative is converted into a pharmaceutical preparatiion, if desired, after freeze drying and subsequent reconstitution in pharmaceutical form.

16. A process according to Claim 15, in which the content of the derivative in the pharmaceutical preparation is clinically comparable with conventional pharmaceutical preparations of the parent bovine or porcine insulin.

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Agent for the Applicants

Printed for Her Majesty's Stationery Office by The Tweeddale Press Ltd., Berwick-upon-Tweed, 1973.
Published at the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

1343113
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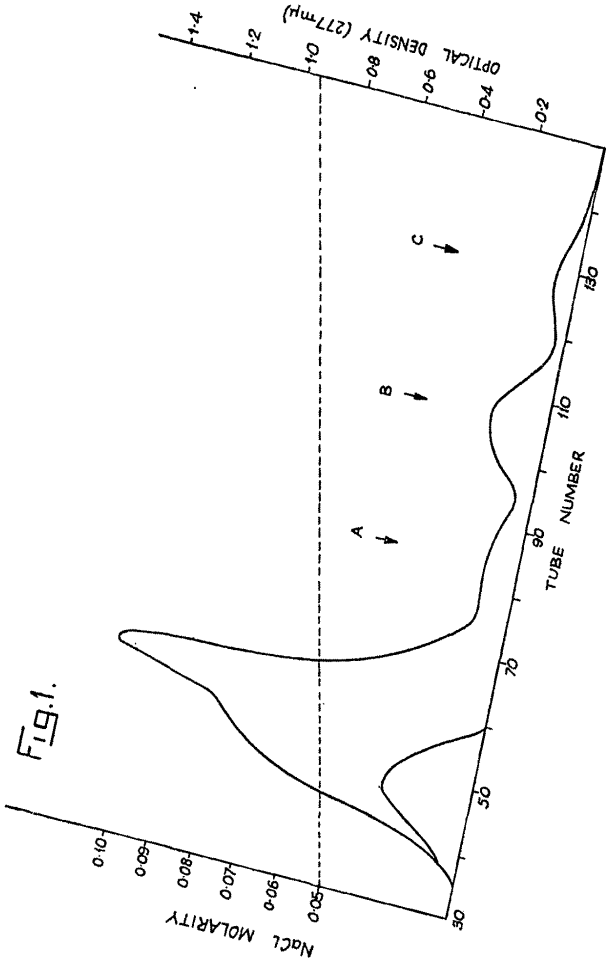

Fig.1.

1343113
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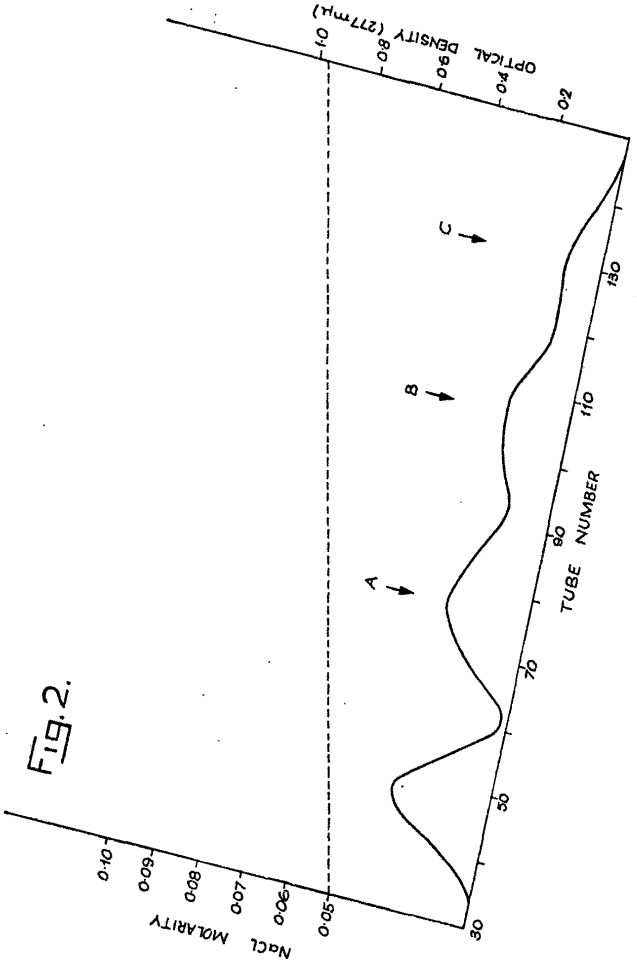

Fig. 2.

1343113 COMPLETE SPECIFICATION
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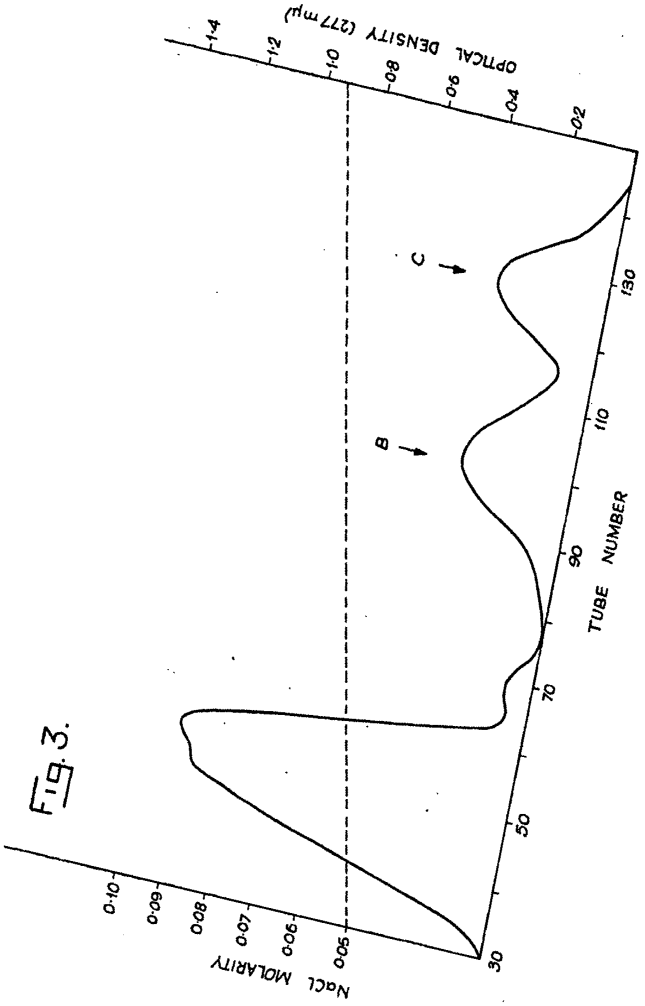


Fig. 3.

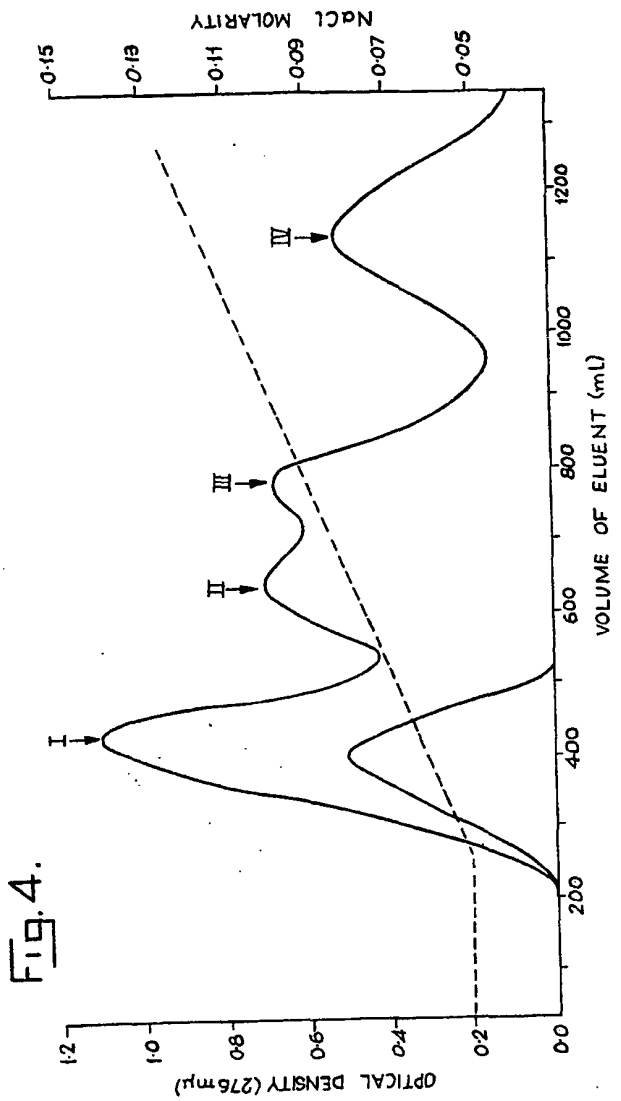
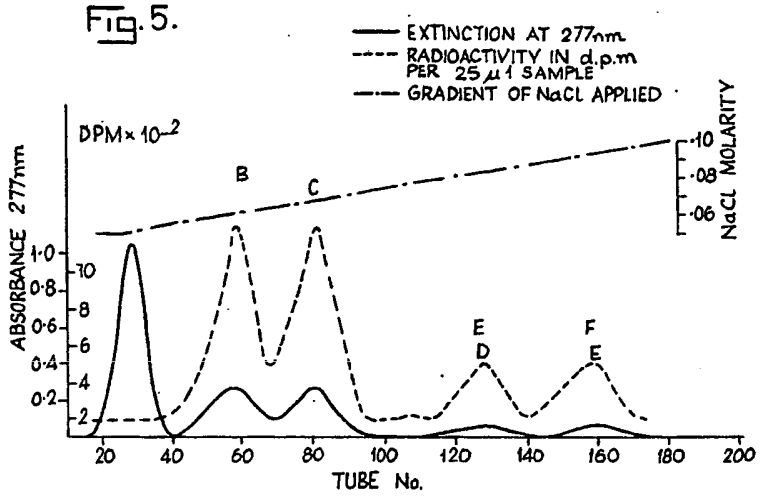

NaCl MOLARITY
0·15
0·13
0·11
0·09
0·07
0·05
OPTICAL DENSITY (276mμ)
1·2
1·0
0·8
0·6
0·4
0·2
0·0
VOLUME OF ELUENT (ml)
200
400
600
800
1000
1200
I
II
III
III

Fig. 4.

Fig.5.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RADIOACTIVITY IN d.p.m PER 25μl SAMPLE
GRADIENT OF NaCl APPLIED
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ABSORBANCE 277nm
NaCl MOLARITY
B
C
D
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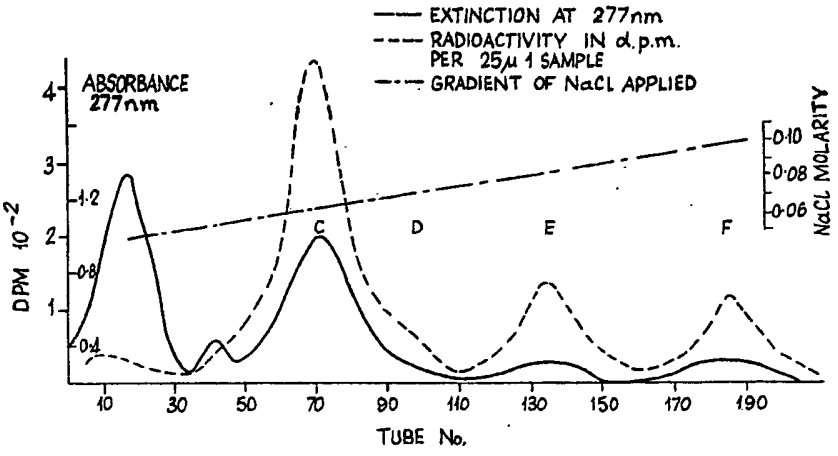
TUBE No.

Fig.6.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NaCl MOLARITY
C
D
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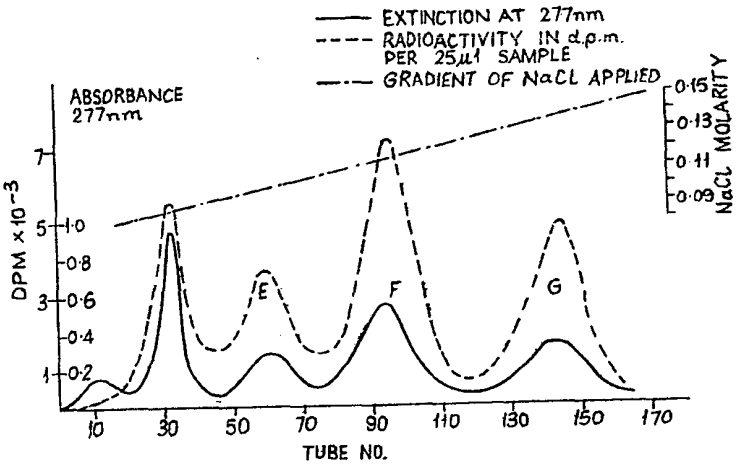
TUBE No.

Fig. 7.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GRADIENT OF NaCL APPLIED
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NaCL MOLARITY
E
F
G
TUBE NO.